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In Vitro Immunotoxicological Assays for Detection of Compounds Requiring Metabolic Activation

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A system for metabolic activation of cyclophosphamide (CP), consisting of a crude microsomal fraction of mouse liver and necessary cofactors (S9 mix), was interfaced with three murine cell culture assays for immunotoxicity. These assays were: the Mishell-Dutton assay for *in vitro* antibody formation, splenic lymphocyte responsiveness to mitogens and bone marrow cell cultures. There was no effect of CP at doses up to 261 μg/ml (1*mM*) on any of the parameters measured unless S9 mix was included. Much greater potency was achieved if the S9 mix was prepared from livers of mice pretreated with phenobarbital. Under these conditions a dose-related inhibition of plaque-forming cells (PFC) in the Mishell-Dutton assay was observed, yielding an ED_{50} of 6.3 μg/ml. When splenic lymphocytes were exposed to CP in the presence of induced S9 mix, a dose related inhibition of the response to the B-cell mitogen, lipopolysaccharide (LPS), and to the T-cell mitogen, concanavalin A (Con A), was observed. For the optimum LPS concentration, the ED_{50} for CP was 8.1 μg/ml; for the optimum concentration of Con A, the ED_{50} was 6.7 μg/ml. DNA synthesis was not inhibited by the doses used. When bone marrow cells were exposed to CP in the presence of induced S9 mix, the stem cell population, enumerated by colonization in semisolid medium, was reduced in a dose-dependent manner, with an ED_{50} of 5.2 μg/ml. Again, DNA synthesis was not affected unless higher doses of CP were used.

In vitro assays for defining toxicological effects are becoming increasingly important as the burden of necessary investigations increases. Our laboratory has successfully interfaced a system for metabolic activation with several *in vitro* assays for immunotoxicity, thus widening the scope of these assays to include immunosuppressive agents which are not direct acting. Cyclophosphamide (CP) was chosen as a positive control in these assays since it requires metabolic activation (*1*) and is immunosuppressive in the mouse (*2*).

Metabolic Activation System

A crude microsomal fraction was prepared from livers of male BALB/c mice to serve as a source of enzymes for metabolic activation. In some cases, the enzymes were induced with phenobarbital by placing 1 mg/ml in the animals' drinking water for 1 week until 24 hr prior to sacrifice by cervical dislocation. Livers were removed aseptically and homogenized in 3 ml of cold, sterile 1.15% KCl/g liver. This homogenate was centrifuged at 9000*g* and the supernatant (S9 fraction) removed and frozen in 1 ml aliquots. When used, a tube of S9 was removed from storage at −70°C, thawed and added to a solution of cofactors. This S9 mix contained 3 mg S9 protein, 2 *mM* NADP, and 35 *mM* isocitrate in sterile culture medium and was added directly to cell suspensions, 0.1 ml/ml of cell suspension.

Mishell-Dutton Assay

The Mishell-Dutton *in vitro* antibody-producing assay has been found useful for recognizing immu-

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Table 1. *In vitro* inhibition of the primary antibody response to sheep erythrocytes by cyclophosphamide in the Mishell-Dutton assay.[a]

Cyclophosphamide					
mM	μg/ml	S9 Mix	PFC/culture	10^6 Cells	PFC/10^6 cells
None	0	None	1760 ± 210	5.55 ± 0.47	317
1.0	261	None	1707 ± 154	5.27 ± 0.17	324
None	0	Naive	2520 ± 218	5.00 ± 0.23	504
0.1	26	Naive	1807 ± 140	4.12 ± 0.59	439
0.3	78	Naive	1035 ± 121	5.02 ± 0.81	206
1.0	261	Naive	727 ± 144	2.92 ± 0.34	249
None	0	Induced	2227 ± 154	5.05 ± 0.41	441
0.01	2.6	Induced	1835 ± 161	3.62 ± 0.53	507
0.03	7.8	Induced	795 ± 111	3.65 ± 0.18	218
0.10	26	Induced	None	1.88 ± 0.23	0

[a]Spleens were obtained from male BALB/c mice, 10 weeks of age. Plaque forming cells (PFC) were enumerated on day 5 of culture and are expressed as the mean ± standard error (n = 3). Background (no antigen) was 102 ± 23 PFC/culture.

nosuppressive chemicals (*3*). In addition to specificity, the system offers the advantage of complex cellular interactions involved to achieve the ultimate response. The disadvantage of the assay, as pointed out by Kutz et al. (*4*), is that chemicals which require activation to reactive metabolites are not detectable. With the modification described here, chemicals which require metabolic activation can be tested in the assay.

The modified assay was carried out by first preparing a spleen cell suspension in RPMI 1640 culture medium and adjusting the cell concentration to 1.5×10^7 cells/ml. The suspension was then distributed into 10 × 35 mm plastic petri plates, 1 ml per plate. To each plate was added 0.1 ml of S9 mix and CP (Sigma) as indicated in Table 1. The plates were incubated at 37°C for 1 hr in a culture box gassed with a mixture of 10% CO_2, 7% O_2, 83% N_2 on a rocker platform.

As described elsewhere (*5*), the 1 hr preincubation was necessary because of loss of response of the assay when S9 mix was added at the time of antigen and left in for the 5 days of culture. Following this incubation period, the contents of each plate were transfered to a sterile plastic tube and centrifuged at 2000*g* for 10 min. Each pellet was resuspended in 1 ml RPMI 1640 (1×10^7 cells/ml), 0.1 ml fetal calf serum and antigen (5×10^6 sheep erythrocytes) were added, and 1 ml of the resulting suspension was placed in a fresh petri plate. The plates were then placed in the culture boxes, gassed with the special mixture, and incubated at 37°C with gentle rocking and daily feeding as described by Mishell and Dutton (*6*). On day 5, the contents of each plate were collected for enumeration of plaque-forming cells (PFC) using Cunningham slides (*7*). Cell counts were performed by using a Model ZBI Coulter counter.

The effect of CP on the primary antibody response of splenic lymphocytes to sheep erythrocytes using the modified Mishell-Dutton assay is shown in Table 1. The control value (1760 PFC/culture) can be compared to a mean control value from six experiments not involving the modification of 2680 ± 273. Cyclophosphamide had no effect up to 1*mM* (261 μg/ml) when S9 mix was not included, but produced a dose-dependent inhibition in the presence of S9 from livers of either naive or phenobarbital-induced mice. The use of the induced preparation caused a tenfold increase in potency of CP, as would be expected. The preparations used in this experiment had been stored for 3 months at –70°C. Although the fractions were more active when used shortly after preparation, the induced preparation was slightly inhibitory at that time.

There is an effect on cell yield at the higher doses of CP when S9 mix is included which seems to be related to the reduced yield of PFC/culture. For comparison, PFC/10^6 cells is shown in the last column of Table 1. Approximate ED_{50} values for CP when activated by induced S9 are 6.3 μg/ml with PFC/culture as endpoint and 7.8 μg/ml with PFC/10^6 cells as endpoint.

Splenic Lymphocyte Cultures

We have been using murine splenic lymphocyte cultures to assess immunotoxicity of a variety of chemicals. The parameters measured include effects on DNA synthesis and response of the lymphocytes to mitogens. The use of the S9 mix and effect of CP in this assay will be described.

Cell suspensions were prepared in RPMI 1640 by using spleens from male CD-1 mice, a random-bred albino from Charles River, which were 10-12 weeks of age. This strain is also being used in our laboratory for *in vivo* toxicological studies. Following collection of the cells by centrifugation, the pellet

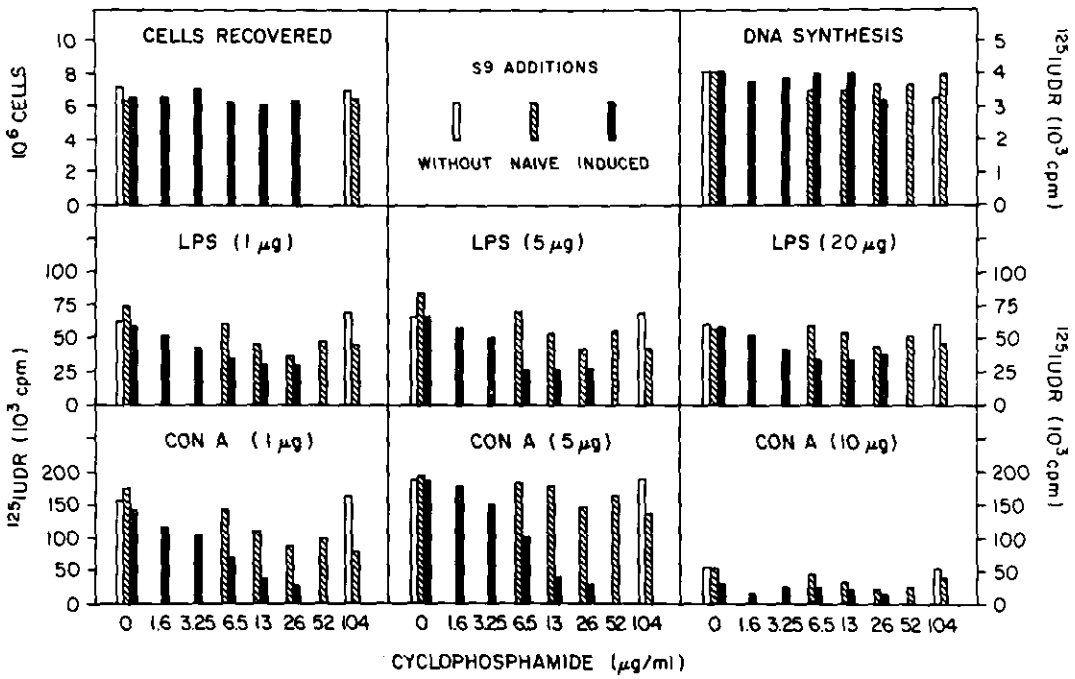

FIGURE 1. Effect of cyclophosphamide on cultured murine splenic lymphocytes. Suspensions of lymphocytes were exposed for 1 hr to cyclophosphamide at the doses indicated. In some cases S9 mix from either naive or phenobarbital induced mouse livers was included, as indicated by hatched or solid bars, respectively. The values shown are means from triplicate exposures.

was resuspended in RPMI 1640 containing 10% calf serum and adjusted to 5×10^6 cells/ml. Cyclophosphamide and S9 mix were added to triplicate siliconized glass tubes, each containing 5 ml of cells. A 1-hr incubation was performed with the tubes in a roller drum in a 37°C, 5% CO_2 incubator. At the end of this incubation, the cells were collected by centrifugation and resuspended in RPMI 1640 containing 10% fetal calf serum for counting. Viability was determined by trypan blue exclusion.

For determination of DNA synthesis, a portion of the cell suspension was adjusted to 3×10^6 cells/ml and incorporation of ^{125}I-iododeoxyuridine (IUdR) was measured in the presence of 1 μM fluorodeoxyuridine (FUdR). In this assay, 6 replicate 200 μl aliquots were incubated with label in a microtiter plate for 1 hr, and the cells were collected with a Titertek Cell Harvester. Quantitation of ^{125}I was by gamma counter.

The response of splenic lymphocytes to the B-cell mitogen, lipopolysaccharide (LPS) from Salmonella, and the T-cell mitogen, concanavalin A (Con A), was determined by IUdR incorporation also. Suspensions were adjusted to 5×10^6 cells/ml and six 100 μl replicates incubated with mitogen in microtiter plates in a 37°C, 10% CO_2 incubator for 48 hr. Three concentrations of each mitogen were used. The samples were then pulsed for 18 hr with ^{125}IUdR/FUdR and the ^{125}I incorporation determined as described for DNA synthesis.

Table 2. DNA synthesis in spleen cells exposed to cyclophosphamide in the presence of S9 mixes.

CP, μg/ml	^{125}IUdR incorporation, cpm/6 × 10^5 cells[a]		
	Without S9 Mix	Naive S9 Mix	Induced S9 Mix
0	4093 ± 128	4109 ± 90	4089 ± 273
1.6	ND	ND	3791 ± 256
3.25	ND	ND	3897 ± 75
6.5	ND	3515 ± 236	4026 ± 200
13	ND	3460 ± 194	4020 ± 201
26	ND	3742 ± 203	3160 ± 76
52	ND	3680 ± 101	ND
104	3607 ± 600	4045 ± 214	ND

[a]Means ± standard error (n = 3); ND = not done.

Cell recovery was not affected by CP, even when S9 mix was included. This is illustrated graphically in Figure 1. Viability, which was 91% in the control was also not changed by any of the treatments. DNA synthesis was also unaffected, as shown in Table 2 and Figure 1. Data from other experiments which are not shown here indicate that CP in the presence of induced S9 inhibits DNA synthesis at higher doses than used in the experiment described.

Mitogenicity data are shown in Table 3 and Fig-

Table 3. Spleen cell response to mitogens in the presence of cyclophosphamide (CP) or S9.

Mitogen	^{125}IUdR incorporation, 10^3 cpm/5 × 10^5 cells[a]			
	Without S9, no CP	Without S9, CP = 104 μg/ml	Naive S9, no CP	Induced S9, no CP
LPS				
1 μg/well	63.7 ± 3.3	69.8 ± 2.9	73.3 ± 0.3	67.7 ± 3.4
5 μg/well	66.0 ± 3.6	68.9 ± 0.8	85.2 ± 7.8	67.4 ± 3.9
20 μg/well	60.3 ± 4.3	61.0 ± 1.8	63.7 ± 2.9	58.9 ± 3.0
Con A				
1 μg/well	155.9 ± 5.4	161.1 ± 2.6	173.1 ± 2.2	138.3 ± 5.8
5 μg/well	188.4 ± 2.7	188.4 ± 2.7	198.8 ± 4.9	181.0 ± 6.6
10 μg/well	55.4 ± 3.2	51.1 ± 3.8	51.1 ± 3.2	27.7 ± 10.8
None	1.5 ± 0.2	1.9 ± 0.2	1.7 ± 0.1	2.1 ± 0.2

[a]Mean ± standard error (n = 3).

ure 1. Table 3 gives the control values, and Figure 1 is a graphic representation of mitogen responsiveness when both CP and S9 are included in the 1-hr preincubation. A dose-dependent inhibition by CP is observed when S9 is included, and this inhibition occurs at lower doses if S9 from phenobarbital induced animals is used as the source of activating enzymes. The ED_{50} in this case is 8.1 μg/ml for LPS (5 μg/well) and 6.7 μg/ml for Con A (5 μg/well).

Bone Marrow Cultures

Immediately after removal of spleens from CD-1 mice, the hind limbs were removed and placed on ice. Using aseptic techniques, the femurs were trimmed and the contents flushed into a plastic centrifuge tube with α MEM (minimum essential medium) containing 10% fetal calf serum and adjusted to 3 × 10^6 cells/ml. Aliquots of 5 ml were placed in siliconized glass tubes, S9 mix and CP added where indicated, and the tubes incubated 1 hr in the roller drum as described for splenic lymphocytes. Cells were then collected by centrifugation and resuspended in α MEM with 10% fetal calf serum. Cell concentration and viability were determined, then the cells were adjusted to 3 × 10^6 cells/ml for determination of DNA synthesis as described for lymphocytes.

Viable stem cells were then enumerated by colonization in semisolid medium. For this determination, the cells were adjusted to 10^5 cells/ml in α MEM-containing 10% fetal calf serum, 5% horse serum, 1.8% methyl cellulose, and 10% L-cell conditioned medium. A 2-ml portion of suspension was placed in each of three wells in a six-well Linbro plate, and the plates incubated at 37°C, 10% CO_2, 95% humidity for 7-10 days. Colonies, defined as clusters of 25 cells or more, were then counted.

The results of a typical dose response experiment using S9 from both naive and phenobarbital induced animals are given in Table 4. Cell yield was not affected by the treatment; viability was 91-93% in controls as well as all high dose samples. As shown in Table 4, there was no inhibition of DNA synthesis at any dose of CP, even when S9 was included. Colony formation was affected; however, in a dose related manner when S9 from either naive animals or phenobarbital induced animals was included. Approximate ED_{50} values for the CP effect on stem cells are 20.8 μg/ml for uninduced S9 and 5.2 μg/ml for induced S9.

Discussion

In vitro activation of CP was first described in Ames' Salmonella microsome test for mutagenicity

Table 4. Effect of cyclophosphamide on DNA synthesis and stem cells in bone marrow cell cultures.[a]

CP, μg/ml	DNA Synthesis, 10^3 cpm ^{125}I			Stem cell enumeration, colonies/10^5 cells		
	Without S9	Naive S9	Induced S9	Without S9	Naive S9	Induced S9
None	33.5 ± 4.0	24.6 ± 3.1	23.2 ± 0.9	163.4 ± 1.0	163.4 ± 0.9	161.1 ± 0.9
3.25	ND	ND	27.0 ± 2.2	ND	ND	104.1 ± 0.7
6.5	ND	24.3 ± 1.3	31.3 ± 2.0	ND	142.4 ± 1.0	66.9 ± 1.5
13	ND	25.7 ± 1.1	33.2 ± 2.5	ND	123.7 ± 1.5	29.3 ± 2.2
26	40.6 ± 3.9	33.7 ± 2.1	ND	162.7 ± 2.2	51.6 ± 2.0	ND

[a]All values represent the mean ± standard error (n = 3). ND = not done.

Table 5. Summary of ED_{50} values for cyclophosphamide activated *in vitro* by S9 mix from phenobarbital-induced mice.[a]

Assay	End point	ED_{50}, μg/ml
Mishell-Dutton	PFC/culture	6.3
	PFC/10^6 cells	7.8
Spleen lymphocyte	Response to LPS (5 μg/well)	8.1
	Response to Con A (5 μg/well	6.7
	DNA synthesis	>26
Bone marrow	Stem cell	5.2
	DNA synthesis	>26

[a]The values shown here were obtained from log dose plots of data shown in the other tables.

(*8*). Since then, it has been used as a model compound in other, more sophisticated mammalian *in vitro* assays. Stetka and Wolff were able to induce sister chromatid exchanges in cultured Chinese hamster ovary cells by including rat liver microsomes with CP (*9*). Recently, an *in vitro* teratogenic assay was described in which CP induced defects when an hepatic microsomal fraction was included (*10*). The present report extends use of *in vitro* activation of CP into the area of immunotoxicology.

A system for metabolic activation of CP *in vitro* has been successfully interfaced with three cell culture assays for immunotoxicity. A summary of the effects of CP when activated by an S9 mix from phenobarbital induced animals is given in Table 5. The approximate ED_{50} values for specific end points in the three assays are very similar, ranging from 5.2 to 8.1 μg/ml or 2.0 to 3.1 $\times$ $10^{-5}M$. It is important to note that DNA synthesis is inhibited only when much higher doses of CP are used. The utility of the described assays is tremendously increased by inclusion of the S9 mix, in that they can now be used for screening for immunotoxicity, as well as elucidation of mechanisms of action. As with all *in vitro* assays, it is important to keep the information in context with *in vivo* effects.

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